

Exhibit 6

Selection for Tn10 Tet Repressor Binding to tet Operator in *Escherichia coli*: Isolation of Temperature-Sensitive Mutants and Combinatorial Mutagenesis in the DNA Binding Motif

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ABSTRACT

We have constructed a genetic assay which selects positively for a functional interaction between Tet repressor and its cognate operator in *Escherichia coli*. In this strain Tet repressor blocks expression of *lacI* and *lacZ*. This leads to derepression of a *lacPO* controlled *galK* gene. The strain can be selected by growth on galactose as the sole carbon source and screened for the β -galactosidase phenotype. These features allow the identification of one candidate among 10^4 false clones on a single plate. The assay was applied to select mutants with a *ts* DNA binding phenotype and to screen oligonucleotide generated Tet repressor mutants. Analysis of these mutations revealed that they affect DNA and inducer binding and possibly the dimerization domains. These mutations are located at residues 21, 48, 49, 89 and at the C terminus of the protein (193), respectively.

THE *tet* determinant on transposon Tn10 confers high level resistance to tetracycline in *Escherichia coli* and other enteric bacteria (FOSTER, HOWE and RICHMOND 1975; KLECKNER *et al.* 1975). Expression of resistance is regulated very tightly at the level of transcription (BECK *et al.* 1982). The regulatory region contains the *tet* promoters as well as two *tet* operators O₁ and O₂ (BERTRAND *et al.* 1983) which are bound by Tet repressor preventing transcription (HILLEN *et al.* 1983; WRAY and REZNICKOFF 1983; MEIER, WRAY and HILLEN 1988). The inducer tetracycline binds to Tet repressor leading to the loss of DNA binding activity. A special feature of this system is the opposite orientation of the *tetR* gene encoding Tet repressor relative to the resistance gene. *tetR* is transcribed by promoters within the *tet* regulatory region and is subject to autoregulation (BERTRAND *et al.* 1983; HILLEN, SCHOLLMER and GATZ 1984). These features are summarized in Figure 1.

Three essential Tet repressor functions are depicted in the figure: dimerization to form the active DNA binding form, DNA recognition and induction by tetracycline. *tet* operator binding probably makes use of an α -helix-turn- α -helix supersecondary structure (ISACKSON and BERTRAND 1985) and several mutants lacking inducibility by tetracycline have been mapped between amino acids 64 and 107 of the 207 amino acid primary structure (POSTLE, NGUYEN and

BERTRAND 1984; SMITH and BERTRAND 1988). We are interested in studying the functional basis of Tet repressor activities and describe in this article the construction, efficiency and application of an *E. coli* strain that allows positive selection for functional Tet repressor-*tet* operator binding. Similar approaches have been used to analyze other protein-DNA recognition reactions (for example see ELLEDGE *et al.* 1989).

MATERIALS AND METHODS

Bacteria and phage: All bacterial strains are derived from *E. coli* K12. Strain K1291 (*pro galK2 rpsL srl::Tn10*) is a derivative of *E. coli* N99 and was obtained from B. RAK, Freiburg, Federal Republic of Germany. This strain was transduced to a *pro⁺AlacX74* genotype using a P1 lysate grown on *E. coli* X7029 (BECKWITH and SIGNER 1966). The resulting *E. coli* strain WH205 was then transduced to *srl⁺* by a phage T4GT7 lysate (WILSON *et al.* 1979) derived from *E. coli* N100 (MCKENNEY *et al.* 1981). This yielded strains with a *srl⁺ Tc^r* phenotype. Since *recA* can be cotransduced with *srl* by T4GT7, candidates were analyzed for hypersensitivity to UV. Isogenic strains WH206 (*galK2 AlacX74 rpsL*) and WH207 (as WH206 but *recA*) were obtained which differ phenotypically only in their UV sensitivity. *E. coli* JM101 was used for propagation of M13mp9 phages and derivatives thereof (YANISCH-PERRON, VIETRA and MESSING 1985). Phages λ plac5 (IPPEN, SHAPIRO and BECKWITH 1971) and derivatives were propagated in *E. coli* XA105 (MILLER *et al.* 1977). *E. coli* NK5031 (*Alac50*) was obtained from L. SMITH, San Diego. The lysogenic phage *Alac50* is identical to λ RStet158-50 (SMITH and BERTRAND 1988; L. D. SMITH, personal communication) and carries the wild-type *et* allele as well as a *tetA-lacZ* fusion. Strain KD1067 (DEONEN and COX 1974) was used as a mutator strain for plasmid DNA. Plasmids were constructed and transformed to *E. coli* strains RRIAZM15 (RÜTHER 1982) or X7029 (BECKWITH and SIGNER 1966).

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A. Wissmann et al.

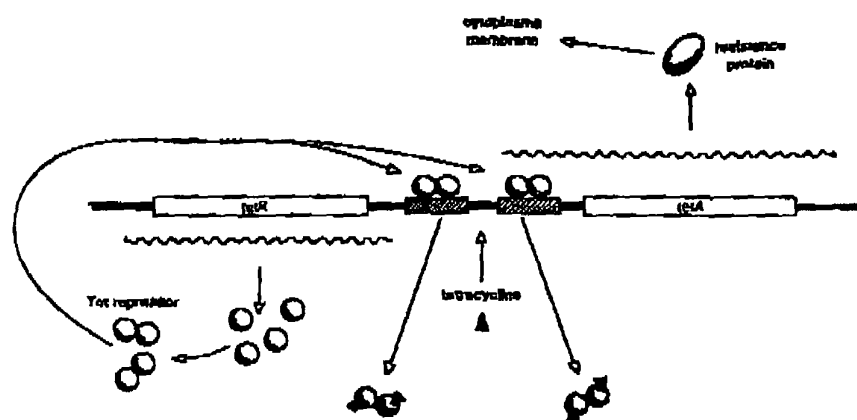


FIGURE 1.—Regulation of gene expression of the transposon Tn10-encoded tetracycline-resistance determinant. Both genes *tetA* (encoding the resistance protein) and *tetR* (encoding the Tet repressor) are indicated. Their divergent expression is symbolized by wavy lines corresponding to the respective mRNAs. The central *tet* regulatory region consists of several promoters (not shown) and the two *tet* operators *O*₁ and *O*₂ represented by hatched boxes. Tetracycline is indicated by the small rectangle which binds to and induces Tet repressor. The Figure was adapted from WISSMANN and HILLEN (1989).

DNA sequence analyses: Single-stranded M13 DNA and double-stranded plasmid DNA were sequenced according to the method of SANGER, NICKLEN and COULSON (1977) and HATTORI and SAKAKI (1986).

Determination of β -galactosidase activities: Assays were done exactly as described by MILLER (1972), except that cultures were grown in LB supplemented with the appropriate antibiotics. For induction studies overnight cultures were grown in the presence of 0.1 μ g/ml tetracycline, whereas 0.2 μ g/ml were added to log cultures. All measurements were repeated at least twice.

Media, enzymes and chemicals: Media and general phage techniques have been described (MILLER 1972; MANIATIS, FRITSCH and SAMBROOK 1982). Antibiotics and *o*-nitrophenyl- β -D-galactoside were obtained from Sigma, St. Louis. Restriction endonucleases, *E. coli* DNA polymerase I large fragment, T7 polymerase, calf intestine alkaline phosphatase and T4 DNA ligase were purchased either from New England Biolabs (Schwalbach), Pharmacia (Freiburg), Boehringer (Mannheim) or BRL (Dreieich). ATP, deoxyribonucleoside triphosphates and dideoxynucleoside triphosphates were obtained from Boehringer (Mannheim). [α -³²P]dATP (400 Ci/mmol) was purchased from Amersham (Braunschweig). Oligonucleotides were synthesized using an Applied Biosystems automated DNA synthesizer model 381A.

Molecular techniques: Mutagenesis of Tet repressor positions 46 to 49 was accomplished by mutually primed synthesis of degenerate oligonucleotides as detailed by HILL (1989). The sequence of the oligonucleotide was 5' GCCAGCATGTAAAAATAAGCGGGCCCTGCTCG-ACGCGTCGAGC 3'. Bold letters (bases shown are wild type) indicate that 6–7% each of the three non-wild-type bases were added at these positions during synthesis of the oligonucleotide.

Plasmids: Plasmid pWH410 contains a fusion of the *tet* regulatory region to the *lac* operon (*tetA-lacZ* fusion). It was derived from pMC1403 (CASADABAN, CHOU and COHEN 1980) and allows Δ lac *E. coli* strains to grow on lactose as the sole carbon source. Plasmid pWH414 differs in two aspects from pWH410. First, it carries a *tetR-lacI* fusion (Figure 2). Second, it contains a one base pair frameshift mutation at the fusion of *tetA* and *lacZ*. This renders Δ lac *E. coli* strains unable to grow on lactose. Nevertheless, phenotypical detection of β -galactosidase activity with X-Gal is still possible.

Transdominance was analyzed in strains containing pWH855. This plasmid is a pBR322 derivative in which the

tet regulatory region was deleted yielding pWH806 and the promoterless Tn10 *tetR* gene was inserted resulting in low level constitutive expression (MÜLLER-HILL, CRAFO and GILBERT 1968).

Plasmid pWH1411 was used for the cassette mutagenesis and as a derivative of pACYC177 (CHANG and COHEN 1978) is compatible to plasmids derived from pBR322. It confers resistance to chloramphenicol and contains a constitutively expressed *tetR* gene. To allow cloning of short oligonucleotide cassettes between singular restriction sites, the sequence of the *tetR* gene was altered without changing the encoded protein sequence. pRT240 is similar to pWH1411, except that it confers resistance to kanamycin and contains a wild-type *tetR* gene (BERTRAND et al. 1984; MEIER, WRAY and HILLEN 1988).

The pACYC177 derivatives pWH1200 and pWH1201 (ALTSCHMIED et al. 1988), pUC19 (YANTSCH-PERRON, VIEIRA and MESSING 1985), pWH483 (MEIER, WRAY and HILLEN 1988) and pMc5-8 (STANSKINS et al. 1989) have been described. Plasmid pWH1012 (STZEMORE et al. 1990) with divergent *tetR-galK* and *tetA-lacZ* transcriptional fusions was used for quantitative analyses of Tet repressor binding to *tet* operator *in vivo*.

Phage constructions and crosses: pWH483 was digested with *Nde*I and *Sma*I yielding a 1950-bp fragment with the entire *galK* gene. In addition, this fragment contains a segment of 180 bp with translational stops in all three reading frames 5' of the gene and a λ t⁺ terminator following the 3'-end of *galK*. After filling in the protruding ends the fragment was cloned into *Hinc*II linearized M13mp9. A candidate with *lac* dependent transcription of *galK* was named mWH22. A second *lac* operator with the proposed ideal binding sequence for Lac repressor (SADLER, SASMOR and BETZ 1983) was cloned 19 bp upstream of the start codon for *galK* into the single *Nru*I site of mWH22 yielding mWH25. In this construction palindromic centers of the two *lac* operators are separated by 233 bp. The *galK* construct from mWH25 was recombined into the *lac* sequences present on λ plac5 to yield λ WH25 (YU and REZNIKOFF 1984). Since this phage carries the *cl*^{ts} allele from λ plac5, *E. coli* strains lysogenized with this phage were grown at temperatures below 33°.

The construction of phage λ tet50 has been described (SMITH and BERTRAND 1986). *E. coli* NK5091(λ tet50) was treated with mitomycin C and the resulting phage lysate used to lysogenize *E. coli* WH207.

Selection of temperature-sensitive Tet repressor mutants: Mutagenized pRT240 was transformed to *E. coli*

Tet Repressor Mutants

227

WH207 containing pWH410 and grown to saturation at 42° in minimal medium with lactose. Since pWH410 contains a fusion of the *tet* regulatory region to the *lac* operon this step represents a selection against binding of Tet repressor to *tet* operator. Afterward pRT240 derivatives were isolated and retransformed to WH207(λWH125) containing pWH414. Transformants were grown to saturation at 28° in minimal medium with galactose. Her, cells containing *tet* operator bound by Tet repressor are selected. The pRT240 derivatives were isolated and retransformed to WH207 with pWH410. Again cells were grown to saturation at 42° in minimal medium using lactose. The pRT240 derivatives were isolated, transformed to WH207 with pWH410 and plated on glucose minimal medium supplemented with ampicillin, kanamycin and X-Gal. Plates were incubated at 42° for 2 days and blue colonies transferred to fresh plates containing the identical medium. *lacZ* phenotypes were scored after incubation at 28° for 2 days. *tetR* genes were recloned as *Hinc*II fragments in pUC19. From derivatives with *lac* promoter *tet* fusions, *Eco*RI/*Sph*I DNA fragments containing *tetR* were then inserted into the respective sites of pWH1200 and pWH1201. This yielded two sets of plasmids with pWH1200 derivatives directing a "high," and pWH1201 derivatives directing a "low" level constitutive expression of *tetR* *in vivo* (BERTRAND *et al.* 1984).

RESULTS

Selection of Tet repressor binding to *tet* operator: The selection makes use of the *tet* directed expression of divergently arranged *lacZ* and *lacI* genes. Binding of repressor to the *tet* operators turns off transcription of both genes resulting in *lacZ*⁻ *E. coli* colonies. At the same time, the absence of Lac repressor allows expression of a galactokinase gene driven by the the *lac* regulatory region. This enables the *E. coli* strain to use galactose as the sole carbon source. In the absence of Tet repressor binding to *tet* operators, *lacZ* as well as *lacI* are expressed. Lac repressor binds to the *lac* operators and prevents transcription of *galK*. The cell cannot utilize galactose as the sole carbon source for growth and displays a *lacZ*⁻ phenotype.

The selection system consists of two plasmids and a λ prophage and is depicted in Figure 2. pWH414 makes use of the divergent *tet* regulatory region in that both a *tetR-lacI* transcriptional fusion as well as a *tetA-lacZ* fusion are present on the same plasmid. Tet repressor is supplied in *trans* by a second compatible plasmid (pRT240). The third component of the system is the prophage λWH25 which provides a single copy *lacPO-galK* fusion. The host strain is *E. coli* WH207 and has a *gal* operon with the *galK2* mutation (see MATERIALS AND METHODS).

A qualitative analysis of this system shows that all components behave as anticipated (see Table 1, lines 1 and 2). In the presence of Tet repressor, the strain is *gal*⁺ and *lacZ*⁻ (line 2, galactose alone). In the absence of Tet repressor, the strain is *gal*⁻ (line 1, galactose ± tetracycline; line 2, galactose + tetracycline). In the absence of Tet repressor, *lacI* repression

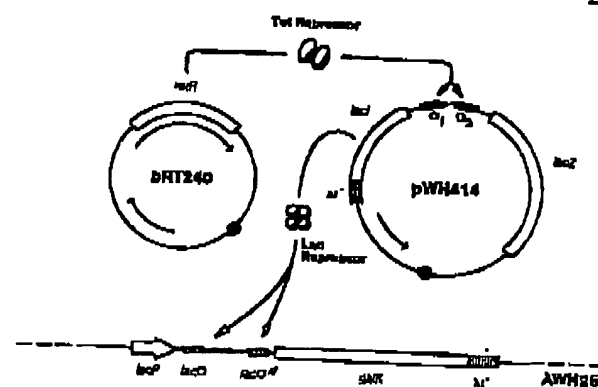


FIGURE 2.—Selection of Tet repressor binding to *tet* operator. DNA is indicated by thin lines, relevant genes as open boxes, the *tet* terminators as stippled boxes, the *lac* operators as hatched boxes, the *tet* operators as filled boxes and the *lac* promoter on λWH25 as an open arrow. Filled circles mark the origins of replication of both plasmids. The arrow expanding through the *tetR* gene in pRT240 indicates the transcript originating from the *tet* promoter, whereas the other arrow defines the kanamycin resistance gene. The arrow in pWH414 indicates the *bla* gene. Tet repressor is shown as a dimer and Lac repressor as a tetramer.

TABLE 1

Tet repressor and Lac repressor dependent expression of galactokinase in *E. coli* WH207(λWH25)

Plasmid	Tet repressor	Growth and phenotype of strains on			
		Glucose	Galactose	Galactose + IPTG	Galactose + tetracycline
pWH414	—	+b	—	+/-b	—
pWH414	+	+w	+w	+w	—
pWH414-2A	—	+b	—	+/-b	—
pWH414-2A	+	+b	—	+/-b	—

Strains of *E. coli* WH207(λWH25) with the indicated plasmids were streaked on minimal plates containing the indicated carbon source and inducer, as well as ampicillin, kanamycin and X-Gal. Plates were incubated at 30° for 3 days and scored for colony growth and color. Abbreviations used are "+" large single colonies, "+/-" small single colonies, "—" no single colonies but very thin bacterial film visible, "w" white colonies and "b" blue colonies. The presence of Tet repressor is indicated by "+" (pRT240), whereas the absence is indicated by "—" (pWH1200). The final concentrations of inducers were 10⁻³ M for IPTG and 0.5 μg/ml (corresponding to subinhibitory amounts) for tetracycline.

can be partially alleviated by addition of isopropyl thiogalactoside (IPTG) (line 1, galactose + IPTG); complete derepression is probably not achieved because Lac repressor is present in such a high amount that it is never fully induced at the IPTG concentration used (10⁻³ M).

We have analyzed the selection system with an operator constitutive mutation to demonstrate the necessity of functional *tet* operators for the observed regulation. For this purpose pWH414-2A was used instead of pWH414 which differs from the latter by a total of 4-bp exchanges in the *tet* operators. MEIER, WRAY and HILLEN (1988) have shown that these mutations reduce binding of Tet repressor by about

three orders of magnitude. The phenotypes in the presence and absence of wild-type Tet repressor are as anticipated (see Table 1, lines 3 and 4). Growth on glucose yields *lacZ*⁺ phenotypes while growth on galactose does not occur irrespective of the presence of Tet repressor. In the presence of galactose and IPTG this strain grows and is *lacZ*⁺ (see above).

For a quantitative determination of the selection efficiency, mixtures of strains were grown on selective plates. These contained cells with the components shown in Figure 2, and an excess of cells in which either the repressor encoding plasmid pRT240 was replaced by the vector without *tetR*, or the wild type operators (pWH414) were replaced by their constitutive mutants (pWH414-2A). The results demonstrate that 30 cells with wild-type Tet repressor and *tet* operator can be efficiently selected on a single plate among 10⁸ cells with either no Tet repressor or the *tet* operator mutation. No white colonies indicating repression of *lacZ* by Tet repressor are selected as false positives from 10⁸ cells. The appearance of a few blue colonies might be due to spontaneous mutations of the *lacI* gene. It is the advantage of the divergent *tet* regulatory region that these candidates can be easily identified and discarded.

Temperature-sensitive Tet repressor mutants: Temperature-sensitive Tet repressor mutations were selected by their ability to confer growth on lactose at 42° and growth on galactose at 28° in appropriate *E. coli* strains (see MATERIALS AND METHODS). Seven parallel selections using individual preparations of pRT240 from the *E. coli* mutator strain KD1067 (DEGEN and COX 1974) were carried through. Five of these selections yielded colonies which were blue at 42° and white at 28° with frequencies ranging from 2 to 85%. The *tetR* genes from one candidate of each of the seven selections were sequenced. The obtained mutations are displayed in Figure 3.

Temperature-sensitive Tet repressor mutants contained either a glycine to glutamic acid exchange at position 21 (GE21) or an isoleucine to asparagine exchange at position 193 (IN193). The latter was independently selected four times. Another mutant (see Figure 3) isolated by a different approach contains an alanine to aspartic acid exchange at position 89 (AD89) and was included in the further *in vivo* analyses. The two mutants without a temperature sensitive phenotype were identical and had a C-terminal deletion (Δ 141). The wild-type and mutant *tetR* genes were re-cloned resulting in two sets of plasmids directing either "high" or "low" level expression of *tetR*.

The mutants were assayed *in vivo* for repression of a *tetA-lacZ* fusion at 28°, 37° and 42°. Furthermore, inducibility by tetracycline and transdominance over wild type was tested. The results are presented in Table 2. Tet repressor mutants GE21, AD89 and IN193 display a clear temperature dependency of *lacZ*

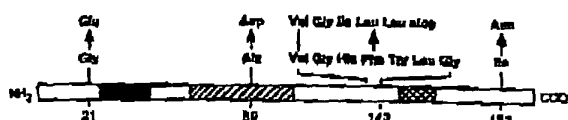


FIGURE 3.—Sequences of mutant Tet repressor proteins obtained from the selection for temperature sensitive variants. The Tet repressor protein with a total length of 207 amino acid residues is represented by a linear bar with both the N- and C-terminal ends indicated. The solid portion defines the potential α -helix-turn- α -helix motif, which is thought to be involved in DNA binding (amino acid residues 26 to 47; ISACKSON and BERTRAND 1985). The region of the protein for which mutants have been obtained that are defective for induction by tetracycline is hatched (amino acid residues 64 to 107; SMITH and BERTRAND 1988). Finally, a region of the protein that shows a high degree of variability when sequences of Tet repressor proteins from the five known resistance classes A through E are compared has been marked by crosshatching (amino acid residues 151 to 168; TOVAR, ERNST and HILLEN 1988). The glycine to glutamic acid exchange at position 21 is due to a transition of G to A, the exchange of isoleucine to asparagine at position 193 is the result of a T to A transversion and the deletion of one G in a run of four Gs leads to a frameshift resulting in a C-terminally deleted Tet repressor protein with a total length of 141 residues. Another temperature sensitive mutant which was isolated by a slightly different procedure (mutagenized pRT240 was transformed to *E. coli* X7029 containing plasmid pWH410 and resulting transformants analysed for their *lacZ* phenotype on X-Gal plates at 28° and 42°; M. GEISSENDÖRFER and W. HILLEN, unpublished results) was also included in the study. This mutant contains an exchange of alanine to aspartic acid at position 89 as the result of a C to A transversion.

repression, as evident from the ratios, whereas mutant Δ 141 does not show repression in this system at all. At 28° and a "high" level of *tetR* expression IN193 shows almost wild-type activity and is clearly more active than AD89. On the contrary at a "low" level of *tetR* gene expression IN193 is not as effective as wild type and is even less active than AD89. The repression efficiencies encoded by the "high" expression plasmids are 95- and 900-fold higher for AD89 and IN193, respectively, than the ones found in the "low" expression plasmids. AD89 is only partially inducible by tetracycline, whereas the other mutants can be fully induced. GE21 and AD89 are transdominant.

Combinatorial mutagenesis at the C terminus of the putative DNA recognition α -helix of Tet repressor: Assuming that Tet repressor contains an α -helix-turn- α -helix motif for operator recognition (POSTLE, NGUYEN and BERTRAND 1984; PABO and SAUER 1984; ISACKSON and BERTRAND 1985), it is very likely that position 46 is part of the α -helix, whereas the secondary structures of residues 47 to 49 remain unclear. To gain information about their possible participation in operator binding a combinatorial cassette mutagenesis (REIDHAAR-OLSON and SAUER 1988) of Tet repressor was performed (see MATERIALS AND METHODS) as shown in Figure 4. Mutant plasmids were transformed to *E. coli* strains that either do or do not allow selection for *tet* operator binding of Tet repressor. *tetR* genes of candidates from both procedures were sequenced in the region of mutagenesis. Thirty-four

Tet Repressor Mutants

TABLE 2

229

In vivo analysis of mutant Tet repressors

Tet repressor	tetR expression	Repression				Tetracycline induction		Transdominance		
		28°	37°	42°	Ratio 37°/28°	-Tc	+Tc	-wt TetR	+wt TetR	Ratio +wt TetR/2.9
None		100.0 (±2.5)	100.0 (±3.4)	100.0 (±5.9)						
Wild type	High	0.0 (±0.0)	0.1 (±0.1)	0.2 (±0.1)	1	100.0 (±5.1)	100.0 (±4.5)	100.0 (±4.1)	2.9 (±0.1)	1.0
GE21	High	18.4 (±2.1)	86.2 (±8.2)	99.3 (±1.2)	4.7	1.9 (±0.2)	98.7 (±2.8)	1.1 (±0.0)	1.1 (±0.0)	0.4
AD89	High	0.8 (±0.1)	43.2 (±3.1)	73.2 (±3.6)	54.0	48.6 (±2.9)	104.8 (±4.7)	94.8 (±1.5)	18.5 (±1.5)	6.4
IN193	High	0.1 (±0.1)	20.3 (±1.8)	69.2 (±1.3)	20.3	4.6 (±1.3)	16.6 (±1.1)	73.4 (±0.2)	5.7 (±0.3)	2.0
Δ141	High	99.4 (±1.9)	104.4 (±6.8)	100.0 (±0.4)	1.1	1.3 (±0.3)	98.7 (±2.4)	9.4 (±0.2)	1.8 (±0.0)	0.6
Wild type	Low	29.7 (±3.0)	51.7 (±1.9)	62.8 (±6.1)	1.7			97.2 (±4.5)	2.5 (±0.2)	0.9
GE21	Low	96.0 (±2.7)	100.1 (±1.1)	100.8 (±3.9)	1.0					
AD89	Low	76.3 (±0.7)	88.3 (±6.9)	93.7 (±0.7)	1.2					
IN193	Low	89.4 (±1.1)	95.1 (±7.8)	97.4 (±4.1)	1.1					

β -Galactosidase determinations were performed in *E. coli* WH207 containing *tetA-lacZ* fusions and plasmids encoding the given Tet repressors. It is indicated whether the "high" or the "low" expression system for Tet repressor was used (see MATERIALS AND METHODS for details). β -Galactosidase values obtained in strains lacking Tet repressor were defined as 100%. "Repression" was measured using plasmid pWH1012 for the *tetA-lacZ* fusion. Actual values obtained were 275.8 (±8.7) units at 28°, 386.1 (±11.0) units at 37° and 341.7 (±15.7) units at 42°. Overnight cultures used for the inoculation of log cultures were also grown at the temperatures indicated, except for measurements at 42°, where overnight cultures were grown at 37°. When overnight cultures of strains containing mutants AD89 and IN193 in the "high" in *vitro* expression system were grown at 28° and log cultures were then grown at 42°, the percentages obtained for AD89 were 37.4% (±3.4%), whereas 2.7% (±0.1%) were obtained for IN193. "Tetracycline induction" was assayed using the prophage λ et50 for the *tetA-lacZ* fusion. Overnight and log cultures were grown at 28°. Actual values obtained in the absence of Tet repressor without addition of tetracycline were 4970 (±585) units and 4125 (±190) units in the presence of tetracycline. "Transdominance" was also determined using the prophage λ et50 as a *tetA-lacZ* fusion. In addition to the indicated plasmids, cells contained a second compatible plasmid which either was pWH806 (indicated by "-wt TetR") or pWH853 (indicated by "+wt TetR"). Details on both plasmids are given in MATERIALS AND METHODS. Overnight and log cultures were grown at 37°. Percentages are related to the 100% value defined by the strain lacking both wildtype and mutant Tet repressors. Typically, 3895 (±160) units were obtained in the absence of Tet repressor.

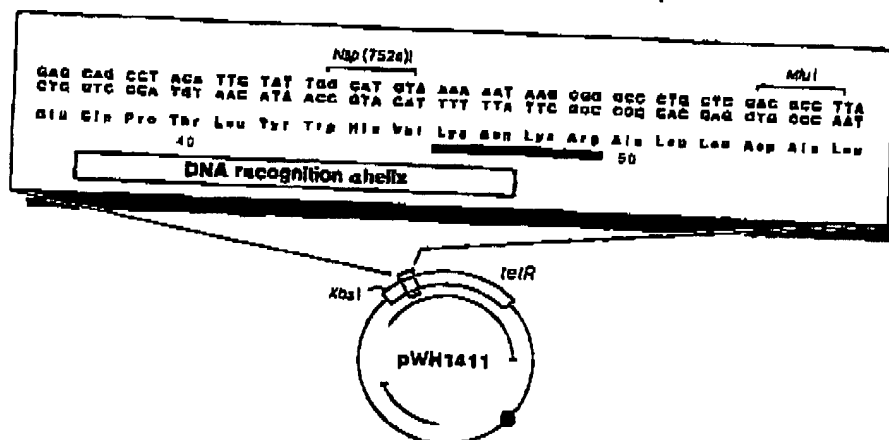


FIGURE 4.—Cassette mutagenesis of positions 46 to 49 of Tet repressor. The DNA of plasmid pWH1411 (see MATERIALS AND METHODS) is shown as a circle with the *tetR* gene emphasized by the open box. The origin of replication is depicted as a filled circle, the chloramphenicol acetyltransferase gene is indicated by the shorter arrow and the transcription originating from the *bla* promoter which leads to constitutive expression of *tetR* is marked by the longer arrow. The orientation of the *tetR* gene is indicated by the *Xba*I site (this restriction site is localized at the 5' end of the gene). At the top of the figure, part of the DNA sequence of the *tetR* gene is shown together with the respective protein sequence. The localization of the potential DNA recognition α -helix is indicated by the open box below the sequence. Amino acid residues at positions 46 to 49 that were altered are underlined by a black bar. Singular restriction sites used for cloning are indicated above the DNA sequence.

different mutants with either single or multiple exchanges at positions 46 to 49 were obtained and analyzed *in vivo* for repression of a *tetA-lacZ* fusion at 28° and 37° and for tetracycline induction.

All mutants isolated with selection for Tet repressor binding to *tet* operator give rise to wild-type *lacZ* repression at 37°. The only exception was a triple

mutant which showed a significant derepression of *lacZ*. At 28°, which was the temperature used for mutant selection, this candidate also displayed wild-type activity.

Single amino acid exchanges at positions 46 and 47 had no detectable effect on repressor activity (data not shown). Three of the five mutants at position 48

TABLE 3
Mutational analysis of Tet repressor positions 46 to 49

Tet repressor	Repression		Induction
	28°	37°	+Tetracycline
None	100.0 (±5.6)	100.0 (±5.6)	100.0 (±2.9)
Wild type	1.7 (±0.1)	1.4 (±0.2)	104.7 (±7.4)
KR48	ND	1.6 (±0.1)	ND
KQ48	5.0 (±0.4)	16.3 (±0.6)	105.7 (±8.2)
KH48	1.8 (±0.2)	3.6 (±1.0)	ND
KM48	ND	1.5 (±0.1)	ND
KT48	15.8 (±0.6)	45.2 (±5.5)	106.2 (±2.1)
RQ49	ND	1.3 (±0.1)	10.3 (±0.3)
RG49	ND	1.4 (±0.2)	ND
RP49	ND	1.3 (±0.1)	32.4 (±1.8)
RW49	ND	1.5 (±0.1)	103.6 (±1.1)

β -Galactosidase determinations were performed in *E. coli* WH207 (Ara150) with plasmids encoding the given Tet repressors. Values are given as percentages with regard to the amount of β -galactosidase measured for this strain containing plasmid pMc5-8 under the specific experimental conditions (specified as "none" in the table). Measurements were carried out with strains grown at 28° and 37°, with the respective overnight cultures grown at the same temperatures. Induction with tetracycline was also done at 37° (for details see MATERIALS AND METHODS).

showed a lower than wild-type repression activity (Table 3). The mutants at position 49 did not affect repression efficiencies but two candidates displayed only partial inducibility by tetracycline. Multiple amino acid exchanges at positions 46 to 49 influenced the repression activity only if position 48 was altered and the tetracycline inducibility only if position 49 was altered (data not shown).

DISCUSSION

Selection of Tet repressor binding to *tet* operator: The selection described above is very efficient, because single cells with wild-type Tet repressor binding to wild-type *tet* operator are found among a vast excess of up to 10^8 cells with either no or reduced binding of Tet repressor to *tet* operator on one plate. The results with the 2A *tet* operator mutation show that Tet repressors must have an association constant of greater than $4 \times 10^8 \text{ M}^{-1}$ to *tet* operator in order to be selectable in this system.

Temperature-sensitive Tet repressor mutants: As depicted in Figure 3, GE21 is located in close proximity to the proposed α -helix-turn- α -helix element. It is the weakest DNA binder and shows the strongest transdominant phenotype of all the mutants analysed in this study. This mutant has been isolated previously by ISACKSON and BERTRAND (1985), but the authors did not describe the temperature dependent effect we have observed. We speculate that this mutation may interfere with the positioning of the DNA binding motif.

AD89 is located in a region where noninducible mutants have been mapped previously (SMITH and

BERTRAND 1988). In agreement with these results it shows only partial induction by tetracycline but also a transdominant phen type. At the same position SMITH and BERTRAND (1988) have also isolated a mutant (alanine to glycine) which allows only partial induction by tetracycline. Since the residue at position 89 affects both the DNA- and the inducer-binding domain it may be involved in structurally transmitting the signal of inducer binding to the DNA recognition domain.

Mutant IN193 is located in the C terminus, to which no function has been assigned so far. It gives rise to the strongest temperature-dependent effect observed in the course of this study. Tetracycline inducibility as far as detectable in our system is not affected and transdominance cannot be observed. When overnight cultures for β -galactosidase determinations were grown at 28° and log cultures were incubated at 42° mutant IN193 retains a much higher efficiency in *lacZ* repression than AD89 (see footnotes to Table 2). This phenotype corresponds to the *ts* ("temperature-sensitive synthesis") mutants first described by SADLER and NOVICK (1965), where the oligomerized protein retains function upon shifting the culture to the non-permissive temperature. Assembly of new dimers is inhibited at the nonpermissive temperature due to either a defect in folding of the monomer or inhibition of dimer formation (GOLDENBERG 1988). This might indicate that IN193 dimers already formed at 28° are not inactivated upon raising the temperature to 42°. On the contrary, it has been shown *in vitro* for mutant AD89 that shifting the temperature to 42° clearly inactivates the protein (B. STADE and W. HILLEN, unpublished results). Western blot analyses have shown identical levels of wild type and IN193 when grown at 28° while at 37° no IN193 protein is detectable (C. BERENS and W. HILLEN, manuscript to be published). Taken together with the large increase in repression with concentration (see Table 2) this leads us to speculate that position 193 of Tet repressor might be involved in dimer formation. The C termini of Tet repressor proteins from five resistance classes are rather homologous. They are preceded by a hypervariable region (amino acid residues 151 to 166 of Tn10 Tet repressor; see Figure 3 and TOVAR, ERNST and HILLEN 1988) which could indicate a possible C-terminal dimerization domain of Tet repressor.

Tet repressor mutants at positions 46 to 49: Several of the Tet repressor mutants at position 48 show reduced DNA binding activity. This suggests that Lys⁴⁸ either directly contacts DNA or that it participates in adjusting the structural conformation of the DNA recognition α -helix. Mutants at position 49 of Tet repressor show wild-type DNA binding, but in some inducibility with tetracycline is reduced. This phenotype can result from three effects: (i) reduced

Tet Repressor Mutants

231

binding of inducer; (ii) interference with the conformational change needed to transmit the signal of inducer binding to the DNA binding domain or (iii) increased affinity for operator resulting in a superrepressor. This effect is not found at the three other positions. The result is particularly surprising, since previously identified mutations in Tet repressor with a noninducible phenotype map between positions 64 and 107 (SMITH and BERTRAND 1988), in a region clearly distinct from the proposed DNA recognition α -helix. The large number of mutants, and a demonstration that some show reduced binding of tetracycline *in vitro* suggest that this region contains the binding site for tetracycline. Thus, Arg⁴⁰ of Tet repressor might be located at the "DNA side" of the switch mediating inducer binding to the DNA binding site. However, superrepression as a result of additional interactions either stabilizing the repressor-operator or destabilizing repressor-nonoperator complexes (HECHT and SAUER 1985) is also possible. In conclusion, the combinatorial mutagenesis suggests that Lys⁴⁸ may be involved in operator binding and Arg⁴⁰ could be active in induction while no functions can be detected for Lys⁴⁶ and Asn⁴⁷.

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